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Baseline

Baseline levels of antioxidant activities in *Mytilus galloprovincialis* along the coast of Cape Town, South Africa

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ABSTRACT

Antioxidant activities in *Mytilus galloprovincialis* were determined from samples collected at Scarborough, Hout Bay, Green Point, Milnerton and Bloubergstrand in Cape Town, South Africa. Antioxidant enzyme activity was determined by measuring CAT, SOD and GSH. The total antioxidant capacity was measured using FRAP and ORAC, while the content and lipid peroxidation marker levels of CDs and TBARS. Antioxidant activities and responses in mussels varied between sites with significant correlations for 85% and 71% of all antioxidant measurements made for Fe and Zn, respectively. The oxidative stress results reported here are novel for the region and indicated that mussels in Cape Town do not have (relatively) high levels of antioxidant activities as a result of exposure to metals. The research undertaken suggests that antioxidant responses was an appropriate biomarker of exposure to metals but more environmental parameters should be considered when interpreting antioxidant responses in the natural environment.

In recent decades, the development of coastal industrial and urban areas has resulted in higher metal contamination of the marine environment (Lima et al., 2007). Monitoring the concentrations contaminants is very important (Galloway et al., 2002; Regoli et al., 2002) and various biomarkers have been postulated to monitor the toxicity of contaminants in the environment (Nasci et al., 2002; Riveros et al., 2002). The choice of biomarkers needs to be related to the source and type of effect of the environment as some metals are known to be carcinogenic (arsenic, chromium and nickel) and the effects of these can be modified by exposure to other metals (Kakkar and Jaffery, 2005). Furthermore, the toxicity of metals (and other contaminants) is dependent on factors such as absorption, distribution, metabolism and excretion which in turn is influenced by the type of metal, form of metal (species), level of exposure, period of exposure, toxicodynamics and toxicokinetics (Kakkar and Jaffery, 2005). Understanding the dynamics of metal toxicity in the environment is complex and this is further compounded when prevalent in the marine environment. Choosing the ideal biomarker to measure the effects of metals on marine organisms, populations or ecosystems are not easy, given the dynamic nature of both the reactivity of metals and the ocean environment.

Antioxidant systems are considered efficient methods to protect organisms against chemical reactive species produced by endogenous metabolism or xenobiotics (Cossu et al., 2000). The reactive species are known as reactive oxygen species (ROS) and are unstable atoms or molecules that try to remove electrons from other molecules to attain more stability and in the process create new free radical species that can cause chain oxidations (Fernández et al., 2010). According to Halliwell and Gutteridge (1984), the use of oxidative mechanisms for metabolism is a continuous source of ROS that results in the univalent reduction of oxygen. It was further noted by Winston and Di Giulio (1991) that pollutants (and their metabolites) could be responsible for harmful effects in organisms by their ability to catalyse ROS.

Cells contain antioxidant enzymes that can detoxify ROS, thereby protecting cells from oxidative injury (Manduzio et al., 2005). The three main antioxidant enzymes to protect the cells against ROS are the superoxide dismutases (SOD), which decomposes $O_2\cdot^-$ to H_2O_2, catalase (CAT) that decomposes H_2O_2 to molecular oxygen and water, and glutathione peroxidase (GPx) which reduces both H_2O_2 and lipid hydrophiles, associated with glutathione (GSH) oxidation (Hebbel, 1986; Almeida et al., 2007; Fernández et al., 2010). According to Fernández et al. (2010), GST breaks down the conjugation of GSH to various forms of electrophilic compounds and hence could be considered to play a role as antioxidants (Prohaska, 1980) performing non-enzymatic defences (Almeida et al., 2007). Glutathione reductase (GR) is not

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considered to play a direct role in getting rid of oxygen radicals. However, it is considered to be an essential antioxidant enzyme because it reduces oxidized glutathione (GSSG) and maintains the GSSG/GSH balance and this is vital for cellular homeostasis and the functioning of other enzymes within the cell (Winston and Di Giulio, 1991; Fernández et al., 2010).

When cell membranes are negatively affected by ROS, membrane lipids initiate an autocatalytic oxidation process called lipid peroxidation (Almeida et al., 2007). When ROS affect cell membranes, lipids are oxidized, resulting in the formation of lipid hydroperoxides (LOOH) (Almeida et al., 2007). Lipid hydroperoxides are also formed via O_2-mediated oxidations (Frankel et al., 1979) and via enzymes such as lipoxygenases (Brash, 1999) and cyclooxygenases (Hamberg and Samuelsson, 1980). The effects of these reactions are that LOOH in membranes disrupts the normal functioning of cellular metabolism, often resulting in adaptive responses and ultimately death (Girotti, 1998; Almeida et al., 2007). The responses to the effects of LOOH are that phospholipid hydroperoxides can either be detoxified directly by phospholipid hydroperoxide glutathione peroxidase (PHGPx) or by the activities of phospholipase A2 and classical glutathione peroxidase (cGPx) (Ursini et al., 1991).

High concentrations of metals in mussels have been shown to induce oxidative stress via the formation of ROS and lipid peroxidation (Viarengo et al., 1990; Almeida et al., 2007). According to Almeida et al. (2007), high concentrations of lipid peroxidation products are correlated with antioxidant enzyme systems (CAT, GPx, GST and SOD) as well as non-enzymatic antioxidants. These authors found that different responses were found in similar experiments, making the interpretation of such results difficult. It has however been accepted that organisms with lowered antioxidant status could be more susceptible to ROS and hence present higher levels of lipid peroxidation (Doyotte et al., 1997; Cossu et al., 2000).

Antioxidant responses (enzymatic and non-enzymatic) and/or lipid peroxidation can be used as a biomarker to measure and monitor oxidative stress caused by exposure to toxicants in the environment (Fernández et al., 2010). Such monitoring is considered an effective means of assessing exposure to, and the effects of pollutants in marine mussels (Regoli and Principato, 1995; Regoli, 1998; Cheung et al., 2002; Lionetto et al., 2003; Box et al., 2007). However, the responsiveness of antioxidants to pollutants is considered difficult to predict due to a high degree of variability in the type of chemicals causing toxicity of ROS, kind of exposure, health of organism as well as phase of life cycle (Livingstone, 2001). Nevertheless, both laboratory and field investigations have shown that variations in antioxidants (levels and/or activities) are potential biomarkers of contaminant-mediated biological effect on organisms (Porte et al., 1991; Regoli and Principato, 1995; Livingstone, 2001).

An experiment by Sparks et al. (2018a) suggested that antioxidant responses in *Mytilus galloprovincialis* in the Cape Peninsula, Cape Town, could be considered as potential biomarkers of metal-induced stress. Hence, the aim of the present study was to conduct a field validation of the laboratory experiment by assessing the potential of antioxidant stress responses as a biomarker of metal-induced stress in *M. galloprovincialis* from five sites along the coast of Cape Town (South Africa). Numerous biochemical activities and responses of the antioxidant system in the mussel were assessed: total antioxidant capacity was determined using ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity assay (ORAC), antioxidant enzyme activity was determined using catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH). Lipid peroxidation was determined using conjugated dienes (CD's) and thiobarbituric acid reactive substances (TBARS). In addition, metals (Mn, Fe, Cu, Zn and Cd) were measured in whole soft tissue of mussels to determine if any correlations between oxidative stress responses and metal concentrations exist.

Samples were collected as per Sparks et al. (2017) (Fig. 1). At the site, the mussels to be used for biochemical analysis were immediately

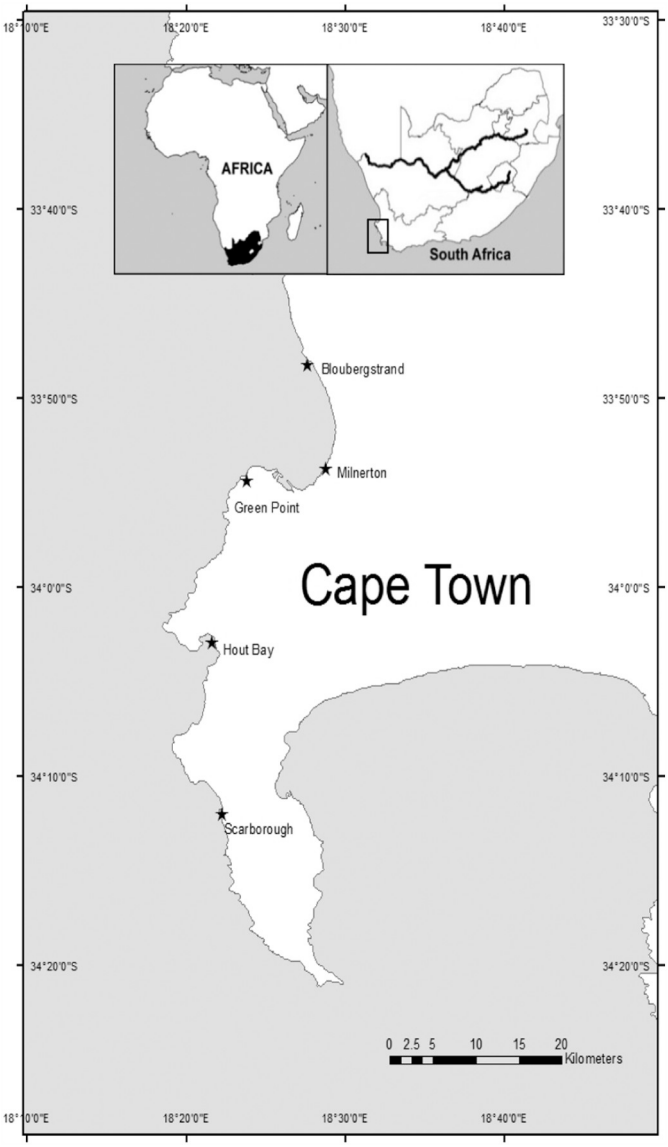

Fig. 1. Map showing the position of the sampling sites in Cape Town, South Africa.

immersed in liquid nitrogen and stored at −80 °C until the analyses were done. In the laboratory, five mussels were pooled per group and freeze dried for 48 h. Thereafter, the soft tissues were combined and split into three aliquots and stored at −20 °C until chemical analyses were done.

Samples were thawed on ice and 0.5 g freeze-dried tissue was added to 5 mL of homogenization buffer and prepared as described by Cheung et al. (2002), with slight modifications. The homogenization buffer contained 50 mM KPO4, 0.1 M KCl, 0.1 mM EDTA, pH 7.4; with 20% glycerol to protect the enzymes. The samples were homogenized using a glass Potter Elvehjem homogenizer and kept on ice throughout the homogenization process until the homogenates were centrifuged at 12,000 × *g* at 4 °C for 30 min. The supernatant was retained and kept at −80 °C for subsequent analysis.

Catalase activity was determined according to Ellerby and Bredesen (2000). The homogenates were thawed on ice and diluted to (1:5 v:v) homogenate to buffer. To a 96-well plate, an assay mixture containing 170 µL phosphate buffer (50 mM KPO4 buffer, pH 7.0) and 5 µL of the homogenate sample, in triplicate, was added. Thereafter, 75 µL H_2O_2 stock solution (30% v/v) was added, the solution mixed well and the decrease in absorbance measured at 240 nm ($\epsilon = 0.00394\ mM^{-1}{\cdot}cm^{-1}$)

in a Multiskan spectrophotometer and the enzyme activity calculated. The results were expressed as mmole/μg protein.

Superoxide dismutase (SOD) activity was determined according to Ellerby and Bredesen (2000). The kinetics of the auto-oxidation of 6-HD was monitored at 490 nm (at 25 °C) for approximately 4 min. The assay was done by adding, in triplicate, 170 μL DETAPAC solution (0.1 mM) in an SOD assay buffer (50 mM, pH 7.5) to a 96-well plate. Samples were diluted 1:10 (v:v) homogenate to buffer and the SOD buffer was added to the wells to make up a final volume of 200 μL. A range of sample volumes were assayed (0, 6, 12, and 18 μL) beforehand and the 6 μL volume of sample was added to the wells. To the DETAPAC and sample solution, 15 μL of stock 6-HD (1.6 mM) was added to initiate the reaction, whereafter the combined solution was mixed and the amount of protein used that resulted in 50% inhibition of auto oxidation of the 6-HD was measured spectrophotometrically in a Multiskan reader at 490 nm. The results were expressed as U/mg protein.

Reduced glutathione (GSH) levels were determined according to Asensi et al. (1999). In this assay, glutathione reductase is added and GSH, which indicates total glutathione presence. GSH determination was done on previously homogenized freeze dried mussels without M2VP. Samples of GSH standards (50 μL) were prepared in triplicate and added to 96-microwell plates. To these wells, 50 μL (0.3 mM) DTNB and thereafter 50 μL of GR (1 u/50 μL) were added. The microwell plates were then mixed and incubated for 5 min at 25 °C. To initiate the reaction, 50 μL of 1 mM NADPH was added to each well and the absorbance immediately measured at 412 nm in a Multiskan reader. The change in absorbance in GSH was determined using a linear function. The results were expressed as μmol/g.

The FRAP assay was carried out as described by Benzie and Strain (1996). The homogenized tissue was thawed on ice and thereafter mixed with 5% PCA (1:1). The PCA mixed sample was centrifuged at 4000 rpm for 5 min at 4 °C. Thereafter, 10 μL (in triplicate) of sample and standards were pipetted to microwell plates. The standards comprised various concentrations (0–1000 μM) of ascorbic acid (AA). Thereafter, 300 μL of FRAP reagent was added to the plates. The FRAP reagent was prepared as follows: 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution, 20 mM $FeCl_3$ solution and distilled water that made up a final volume of 300 μL. The final volume added to the plate was 310 μL. The plate was incubated at 37 °C for 30 min and read at 593 nm in a Multiskan reader. The results were expressed as μmol Ascorbic Acid Equivalents (AAE)/g.

The ORAC method was performed using a fluorescence spectrophotometer until zero fluorescence occurred. The method of Ou et al. (2001) was used on samples that were homogenized as described previously. A 1:10 (5% PCA FRAP homogenate sample: ORAC buffer) diluted sample was used for the ORAC assay. Preparations of the samples were done on ice throughout the whole procedure. The PCA diluted sample was centrifuged at 4000 rpm for 5 min at 4 °C. Thereafter, 12 μL (in triplicate) of sample and standards were pipetted to black 96-microwell plates. The standards comprised various concentrations (0–417 μM) of Trolox solutions. Thereafter, 138 μL of fluorescein was added to the plates followed by 50 μL of AAPH, to initiate the reaction, making up a final volume of 200 μL being added to the wells. The solution in the wells was read using a fluorescence plate reader. The fluorescence of fluorescein was recorded every 5 min for 2 h after the addition of AAPH. The ORAC values were calculated using a regression equation ($Y = a + bx + cx2$) between Trolox concentration (Y) (μM) and the net area under the fluorescence decay curve (x). Data were expressed as micromoles of Trolox equivalents (TE) per milligram of sample (μmol TE/g).

Levels of lipid peroxidation were assessed by measurements of conjugated dienes (CDs) and thiobarbituric acid reactive substances (TBARS). The CDs were measured according to Recknagel and Glende Jr. (1984). To the freeze-dried samples (50 mg), 1 mL of a chloroform:methanol solution (2:1) was added and kept on ice. Solutions were vortexed and then centrifuged at 10,000 rpm for 10 min at 4 °C. The top layer was removed and to the bottom organic layer, 500 μL of HCl was added. This solution was then vortexed for 10 s and then centrifuged at 10,000 rpm for 3 min at 4 °C. From the bottom layer, 100 μL was removed and transferred to a new eppendorf tube and dried under nitrogen gas. To each dried residue eppendorf tube, 1 mL cyclohexane was added. The solution was then placed into micro well plates (in triplicate) and the absorbance measured at 234 nm using a Multiskan reader. The results were expressed as μmol/g.

The TBARS were measured according to Khoschsorur et al. (2000) with slight modifications. The homogenized mussel tissue was thawed on ice and 100 μL added to eppendorf tubes. To the samples, 375 μL of H_3PO_4 (0.44 M) and 125 μL TBA (42 mM) was added and the mixture vortexed for 10 s and heated in a boiling-water bath for 60 min. The solution was allowed to cool on ice for 2 min and then left at room temperature for 5 min. To the cooled solution, 500 μL butanol and 50 μL saturated NaCl was added. The sample was vortexed for 10 s and then centrifuged at 12,000 rpm for 2 min at 4 °C. From this mixture, 150 μL of the supernatant was added to 96 well plates (in triplicate) and the absorbance read at 532 nm in a Multiskan plate reader. The results were expressed as TBARS μmol/μg protein.

Protein concentrations in homogenized tissue samples were determined using a commercially-available protein assay kit (Pierce® BCA Protein Assay Kit, Thermo Scientific). Bovine serum albumin (BSA) was used as the protein standard and quantified by measuring absorbance at 595 nm (Bradford, 1976).

Data were reported as means (± SE). All calculations and data analysis were done using SPSS v25. One way ANOVA was used to ascertain significant differences in antioxidant responses in *M. galloprovincialis* between sites. The data was tested for normality and homogeneity of variance using Shapiro Wilke's and Levene's tests respectively, prior to post hoc comparisons. Post hoc ANOVA analysis were done using the Bonferroni test to determine statistical significances between sites ($p < 0.05$). Pearson's correlations were done on metal concentrations and antioxidant activities in *M. galloprovincialis* to determine relationships between parameters. All significant values were at $p < 0.05$.

To investigate antioxidant activity at different sites, PRIMER V6 was used (Clarke and Gorley, 2006). A principal component analysis (PCA) of the antioxidant activity in *M. galloprovincialis* from the sites sampled was produced. Data were $\log_{(x+1)}$ transformed and Euclidean distance used to produce a resemblance matrix.

The PCA analysis indicated that mussels sampled at Scarborough (and Hout Bay to a lesser extent) were the least affected by antioxidant activity of the all the sites sampled (Fig. 2). Mussels sampled at Green Point were mostly influenced by the effects of CAT and mussels from Bloubergstrand, mostly influenced by FRAP. The combined effects of antioxidant activities were highest at Milnerton, suggesting that mussels at this site had the highest antioxidant activities. There were significant positive correlations between biomarker analysis and Fe (except for FRAP), which suggests that this element could be responsible for the higher antioxidant activities at the sites sampled. Also, Sparks et al. (2018b) measured Fe in mussels from the same sites and reported that Fe concentrations were highest in mussels sampled from Milnerton when compared to the other sites sampled.

The results of the present study provided information about antioxidant status at five sites along the west coast of the Cape Peninsula, Cape Town. Of the sites sampled, Scarborough is considered the reference site as it is situated 40 km south of the city centre, away from any industrial and commercial activity. This site is hence considered to be unpolluted, as it is also adjacent to (< 10 km) the Cape Point Nature Reserve, a national marine park. The low level of metals at that site was also reported in Sparks et al. (2014) and Sparks et al. (2017) supports the postulation for Scarborough to be considered a reference site.

Mean enzyme activity (CAT, SOD and GSH) was significantly highest ($p < 0.05$) in mussels sampled at Milnerton (39.73 ± 0.55 mmole/μg protein, 90.73 ± 4.27 U/mg, 136.15 ± 0.94 μmol/

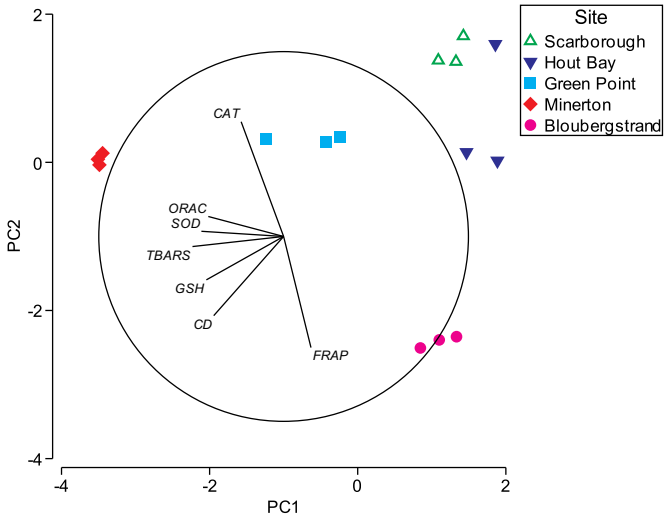

Fig. 2. The PCA analysis of antioxidant activities (CAT, SOD and GSH), antioxidant capacity (ORAC and FRAP) and lipid peroxidation (CD and TBARS) in *M. galloprovincialis*, Cape Town, South Africa.

g, respectively) (Fig. 3). When considering mean antioxidant capacity, Bloubergstrand had significantly highest ($p < 0.05$) FRAP (Fig. 4A) records (2.96 ± 0.03 µmol/g) of the sites sampled and lowest at Scarborough (1.52 ± 0.06 µmol/g) and Milnerton (1.37 ± 0.04 µmol/g). ORAC values were significantly highest in mussels collected from Green Point (29,307 ± 439 µmol TE/g) and Milnerton (28,898 ± 709 µmol TE/g) and lowest in Hout Bay (22,959 ± 722 µmol TE/g) and Bloubergstrand (24,182 ± 335 µmol TE/g) (Fig. 4B).

Lipid peroxidation levels were significantly lowest in mussels sampled in Scarborough (0.55 ± 0.01 mmol/mg protein) and Hout Bay (0.55 ± 0.01 mmol/mg protein) (Fig. 5A) and highest in Milnerton (0.85 ± 0.01 mmol/mg protein). The TBARS levels were lowest at Scarborough (14.09 ± 0.44 µmol/µg protein), Hout Bay (12.91 ± 0.61 µmol/µg protein) and Bloubergstrand (15.45 ± 0.9 µmol/µg protein) (Fig. 5B) and significantly highest at Milnerton (31.67 ± 1.02 µmol/µg protein).

The present study is the first to provide information about the antioxidant activities in *M. galloprovincialis* sampled in the Western Cape, South Africa. According to Santovito et al. (2005), the presence of metal contaminants in mussels can be possible causes of oxidative stress and could have induced various antioxidant responses reported here. Hence, studies that have demonstrated that antioxidant responses can be induced by oxidative stress, suggest that there could be a significant correlation of these responses to pollutant concentrations (Viarengo et al., 1990; Cheung et al., 2002, 2004; De Luca-Abbott et al., 2005; Jena et al., 2009). The results of the present study have also indicated this to be the case. The accumulation of pollutants in bivalves is influenced by a dynamic balance which results in uptake or depuration of these substances and these actions in turn are influenced by a dynamic equilibrium amongst pollutants in sediment, water, food particles and the organisms themselves (Livingstone, 1991).

Both antioxidant enzyme activities and GSH levels suggest that Milnerton is a stressful environment which may have been induced by metal contamination. According to Jena et al. (2009), CAT, SOD and GSH in mussels from polluted sites showed higher activity and levels when compared to sites that were not considered to be polluted. The high variability of CAT at Scarborough could however be as a result of some other stress other than metal pollutants, for example wave action, desiccation, food availability and even other pollutants not measured (Almeida et al., 2007). Scarborough was the first antioxidant station sampled and it is also possible that sampling error could have resulted in the organisms suffering from stress before or while they were being

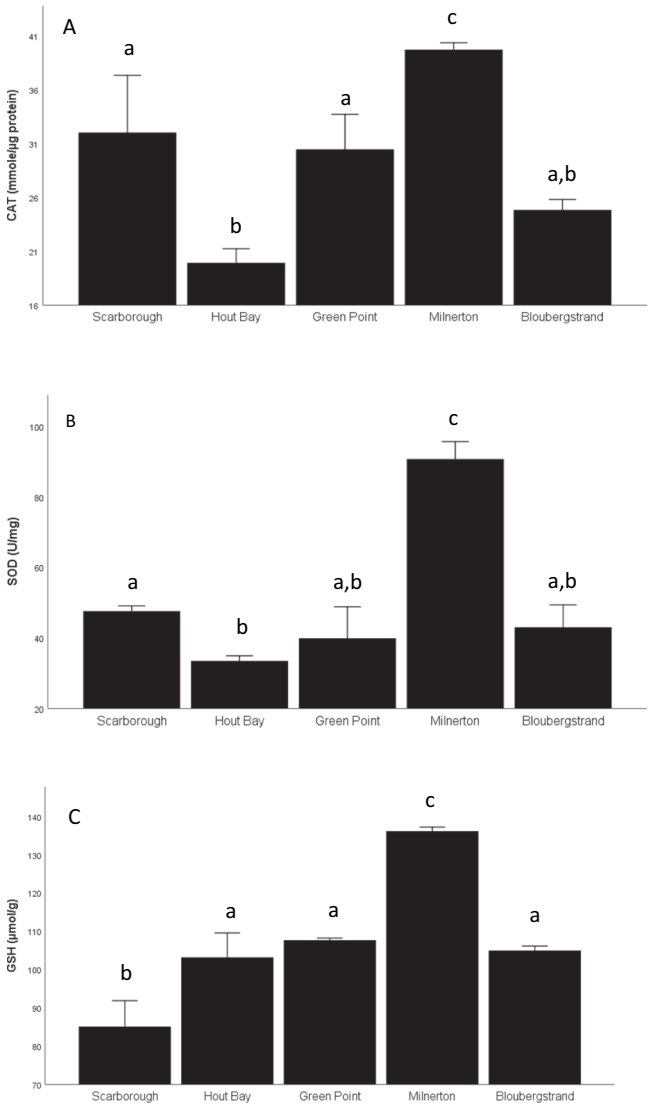

Fig. 3. Mean antioxidant enzyme activities (± SE) in whole soft tissue of *M. galloprovincialis* according to CAT (A), SOD (B), and GSH (C) activities. Similar letters indicate no significant differences between sites (n = 3).

preserved for the antioxidant analysis. Alternatively, a natural factor (higher temperature due to low tide or strong wave action specific to that site) could also have contributed to the higher CAT values recorded at Scarborough. CAT are the primary scavengers of H_2O_2 in the cell and the increased CAT activity recorded in Scarborough (compared to the other sites) could indicate stress (anthropogenic or natural) that resulted in elevated formation rates of H_2O_2. These CAT results reported at Milnerton are comparable to others where CAT activity increased due to exposure to metals (Torres et al., 2002; Jena et al., 2009) and petrochemicals (Verlecar et al., 2008).

Antioxidant capacity (FRAP and ORAC) suggested that Milnerton and Green Point could be considered to be contaminated sites. According to Gorinstein et al. (2006a, b), antioxidant capacity in *M. galloprovincialis* from polluted sites is significantly higher than mussels from non-polluted sites.

According to Jena et al. (2009) mussels with high lipid peroxidation levels are considered to be subjected to stress from contamination. If the same criteria were to be used for the present study, mussels in Milnerton are to be considered under high stress if using both CDs and TBARS as criteria. The lipid peroxidation results further suggest that Scarborough and Hout Bay are not subjected to contaminant-stress if

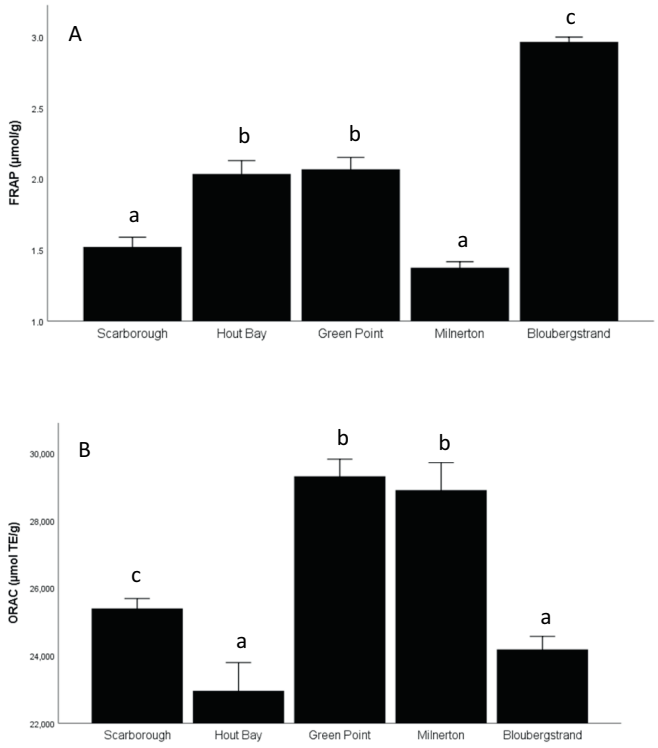

Fig. 4. Mean antioxidant capacity (± SE) in whole soft tissue of *M. galloprovincialis* according to FRAP (A) and ORAC (B) activities. Similar letters indicate no significant differences between sites (n = 3).

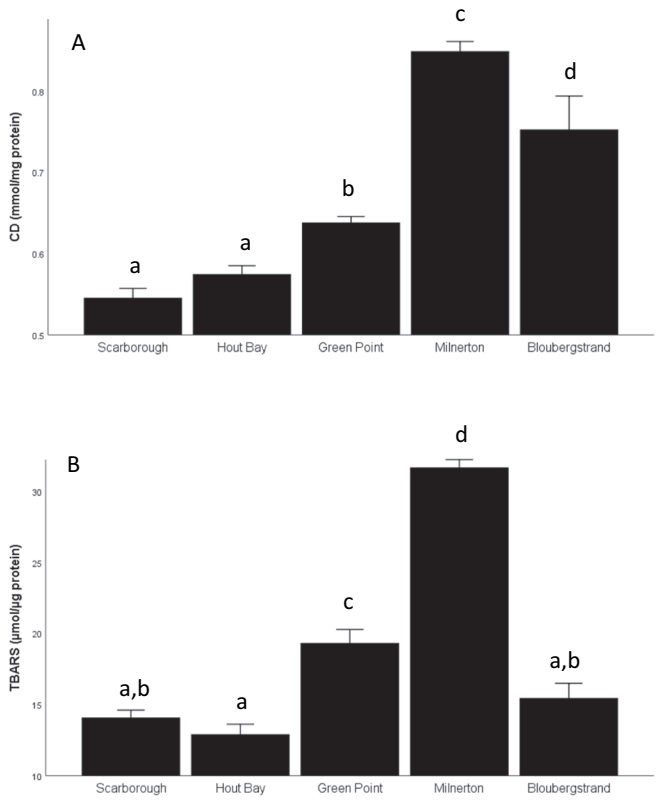

Fig. 5. Mean lipid peroxidation (± SE) in whole soft tissue of *M. galloprovincialis* according to CD (A) and TBARS (B) activities. Similar letters indicate no significant differences between sites (n = 3).

the criteria of Jena et al. (2009) are to be used. Similar results have been recorded elsewhere where an increase in tissue contaminants in bivalves was accompanied by an increase in TBARS (Torres et al., 2002; Cheung et al., 2004).

The MDS (Fig. 2) showed clear discriminations between stressed and unstressed sites. According to Clarke and Warwick (1994) this could also be an indication of contaminated and uncontaminated sites. Box et al. (2007) conducted a study on *M. galloprovincialis* in the Balearic Islands using oxidative stress as biomarkers and found that the MDS analysis allowed for discriminating impacted sites compared to a reference site. The results of that study suggested that antioxidant activities in the polluted sites were more similar and homogenous than cleaner sites where higher dispersion of results was evident. These authors accounted for the higher dispersion by stating that it was due to particular abiotic parameters such as temperature, currents whereas the similarity of polluted sites were due to similar compounds such as metals and persistent organic compounds. If these criteria were to be applied to the present study, antioxidant activities and responses showed that Milnerton could be considered the most contaminated of the sites sampled and Scarborough being least contaminated.

The Milnerton site is at the mouth of a stormwater pipe, situated at shore end of a range of hotels. Within the vicinity is an industrial area, including petro-chemical industries that could have been contributors of contaminants. It was also evident at the time of sampling that the water from the pipe was warmer than the surrounding sea water and this too may have been a contributing factor to the higher antioxidant activities recorded. According to Jena et al. (2009), temperature also plays a role in affecting antioxidant activity in mussels. Scarborough is removed from residential, commercial and industrial activity and hence considered to be not polluted.

Correlations between the metal concentrations and biomarker responses were applied to both biomarkers and metals irrespective of sites (Table 1). Antioxidant activities showed some type of significant correlations with metals, except for Cu where no correlations were recorded and Mn only having a significant correlation with ORAC. CAT was significantly positively correlated with Fe, Zn and Cd, while SOD was significantly positively correlated Fe and Zn. GSH had the least significant positive correlation of the enzyme activities, only being significantly positively correlated with Fe. FRAP was the only biomarker with negative correlations with more than one metal, (Mn, Fe, Cu, Zn and Cd). ORAC were significantly positively correlated with Fe, Zn and Cd, and showed significant negatively correlation with Mn. CD was only positively correlated with Fe, while TBARS showed significant positive correlations with Fe and Zn. The results of the present study are similar to others that measured antioxidant activities in mussels and reported that antioxidant activity/levels were significantly higher in polluted sites than non-polluted sites (Hultberg et al., 2001; Lam and Gray, 2001; De Luca-Abbott et al., 2005; Kakkar and Jaffery, 2005; Gorinstein et al., 2006b). It is therefore proposed that antioxidant responses of *M. galloprovincialis* in the Cape Peninsula be considered as biomarkers of oxidative stress.

The present study provides a preliminary account of antioxidant

Table 1
Summary of Pearson's correlation coefficients between metals in mussels and biomarker analysis. Figures in bold are statistically significant at $p < 0.05$ level. Metal data from Sparks et al. (2018b).

	Mn	Fe	Cu	Zn	Cd
CAT (mmole/µg protein)	−0.267	**0.721**	0.161	**0.640**	**0.573**
SOD (U/mg)	−0.066	**0.746**	0.113	**0.551**	0.289
GSH (µmol/g)	0.228	**0.657**	0.086	0.323	−0.200
FRAP (µmol/g)	−0.013	−0.390	−0.303	**−0.429**	−0.384
ORAC (µmol TE/g)	**−0.477**	**0.574**	0.012	**0.506**	**0.451**
CD (mmol/mg protein)	0.227	**0.600**	0.074	0.278	−0.198
TBARS (µmol/µg protein)	0.044	**0.814**	0.112	**0.568**	0.168

responses in mussels collected from five sites with different hydrographic and water quality status. The results indicated that there are significant differences in antioxidant responses at the five sites sampled suggesting that all the antioxidant activities applied are suitable for consideration as biomarkers of oxidative stress. It is suggested that antioxidant responses in mussels be considered as biomarkers of pollution in South Africa. However, it is recommended that a suite of biomarkers be considered in future research to determine responses to metal induced stress (antioxidant, DNA damage, metallothioneins, lysosomal membrane integrity). The advantage of using a combination of biomarkers is that they integrate effects from various pollutants, and act as an early warning of impacts at the community and population levels (Vasseur and Cossu-Leguille, 2003). More laboratory studies are needed that use cocktails of metals and other pollutants to improve understanding of cause and effect. Such mixture of metals are seen as important measurements of toxicity that provide better reflections of environmental concentrations. Sampling of a variety of potential contaminants should hence be done to correlate the potential causes of higher antioxidant responses at sites that are considered to be polluted. Studies like this will provide essential information in understanding whether antioxidant parameters serve as biomarkers of pollution and whether these could be incorporated into biomonitoring studies.

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Declarations of interest

None.

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